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Short communication

Multiplex detection of *Solenopsis invicta* viruses -1, -2, and -3

Steven M. Valles[a,*], Laura Varone[b], Leonardo Ramírez[b], Juan Briano[b]

[a] *United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural and Veterinary Entomology, 1600 SW 23rd Drive, Gainesville, FL 32608, United States*

[b] *United States Department of Agriculture, Agricultural Research Service, South American Biological Control Laboratory, Bolivar 1559 Hurlingham, Buenos Aires Province, Argentina*

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A B S T R A C T

Multiplex reverse transcription and polymerase chain reaction (PCR) methods were developed to detect *Solenopsis invicta* viruses -1, -2, and -3 simultaneously in their host, the red imported fire ant, *S. invicta*. cDNA synthesis was conducted in a single reaction containing an oligonucleotide primer specific for each virus. Multiplex PCR was subsequently conducted with oligonucleotide primer pairs specific for each virus. The method was specific and sensitive, capable of detecting as few as 500 copies of the viral genomes consistently. Specificity was verified by PCR and amplicon sequencing. The method was evaluated against field-collected samples of ant workers from colonies in Argentina ($n = 135$ ant colonies) and the United States ($n = 172$ ant colonies). The prevalence of each virus in fire ant colonies varied considerably from site to site. A number of colonies exhibited multiple virus infections. However, the multiple SINV infection rate was lower than for single infections. Comparison of viral infection prevalence between *S. invicta* colonies in Argentina and the U.S. showed no statistical differences, regardless of infection category. This method is anticipated to facilitate epidemiological and related studies concerning the *S. invicta* viruses in fire ants.

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Introduced into the United States around 1940 from the Formosa region of Argentina (Caldera et al., 2008), the red imported fire ant, *Solenopsis invicta*, has since spread to infest more than 128 million hectares in the U.S. (Williams et al., 2001). With nest densities as high as 200 colonies per hectare (Macom and Porter, 1996), *S. invicta* is considered the dominant arthropod in infested areas (Vinson and Greenberg, 1986) and has been reported to cause damage exceeding $1 billion annually (Thompson et al., 1995). Although a number of highly effective insecticides are available for controlling this pest ant, sustained control by this method is not practical because chemicals must be applied regularly to maintain areas free of *S. invicta*. Ecological studies have shown that *S. invicta* mound density, mound volume, and population density compared with other ant species in the community are significantly greater in the U.S. than in South America (Porter et al., 1992). These differences have been attributed to a lack of natural enemies and likely explain why *S. invicta* is such a serious pest in the U.S. (Porter et al., 1997). Therefore, a great deal of research effort has been devoted to the discovery, development, release, and use of natural enemies in an attempt to achieve sustained control of *S. invicta* in the U.S. (Williams et al., 2003).

A number of organisms, including parasitoids, fungi, protozoa, and bacteria (Oi and Valles, 2009) have been studied as potential biological control agents against *S. invicta*. However, only recently have viruses been discovered in *S. invicta* (Valles and Hashimoto, 2009; Valles and Strong, 2005; Valles et al., 2004; Valles et al., 2007a). Viruses can be important biological control agents of arthropod pests (Lacey et al., 2001), so there is keen interest in their study and potential use against *S. invicta* (Williams et al., 2003). There are currently three viruses known to infect *S. invicta*. These include *S. invicta* virus 1 (SINV-1), *S. invicta* virus 2 (SINV-2), and *S. invicta* virus 3 (SINV-3); all are positive-strand RNA viruses. Examination and characterization of these viruses are just beginning. Thus, to facilitate epidemiological and related studies, a two-step multiplex PCR method was developed to detect their presence simultaneously. The method was used to examine the prevalence of SINV-1, -2, and -3 in *S. invicta* colonies from areas within the United States and Argentina.

Reverse transcription polymerase chain reaction (RT-PCR) was used to identify ant colonies infected exclusively with SINV-1, -2, or -3 using virus-specific oligonucleotide primers (Valles and Hashimoto, 2009; Valles et al., 2004; Valles et al., 2007a). RNA was extracted from adult worker ants collected from nests around Gainesville, FL, with Trizol reagent according to the manufacturer's instructions (Invitrogen).

Development of the multiplex PCR began by first establishing oligonucleotide primer specificity for each virus with RNA preparations from ants infected with SINV-1, SINV-2, SINV-3, or those uninfected with these viruses. For each RNA prepa-

* Corresponding author. Tel.: +1 352 374 5834; fax: +1 352 374 5818.
E-mail address: steven.valles@ars.usda.gov (S.M. Valles).

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ration, cDNA was synthesized with oligonucleotide primers p524 (5′-ATTGCATTTTCACAATTAATCTTAGTGCTCTC), p548 (5′-TGCATACTCGTTGTAAACAATCTGCTCATCT), and p812 (5′-AATATCAGCATATTGATGATCCAAAATGCCAA). cDNA synthesis was conducted in a 0.5 ml, thin-walled PCR tube containing 1 μl of oligonucleotide primer (1 μM), 0.5 μl (10 mM) of a dNTP mix, 4.5 μl of H_2O, and 50 ng of total RNA (0.5 μl). This mixture was heated to 65 °C for 5 min in a PTC 100 thermal cycler (MJ Research, Waltham, MA), followed by incubation on ice for 2 min. Then, 2 μl of 5× first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM $MgCl_2$), 1.375 μl of H_2O, and 0.125 μl of superscript reverse transcriptase (Invitrogen, 200 units/μl) were added. The mixture was collected by centrifugation and incubated at 55 °C for 30 min, followed by inactivation of the reverse transcriptase by heating to 70 °C for 15 min. To prevent amplification in the PCR reaction from carry-over reverse transcriptase (Craggs et al., 2001), the samples were treated with 0.5 units of RNase A and 5.0 units of RNase H and incubated at 37 °C for 30 min. Each cDNA was used as template for PCR with the following oligonucleotide primer pairs, p524 and p244 (5′-CATCTGGCAATCCTGCAACCAC); p548 and p555 (5′-TGCCGTGACAATCCTGAATATCGTCAGATGTA); and p812 and p813 (5′-AAGAGAACGTATGCCTACTCCATCAGAAGCAT) under the following temperature regime, 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s; 68 °C for 30 s and a final 68 °C step for 5 min in a PTC 100 thermal cycler (MJ Research, Waltham, MA). Amplicons produced were agarose gel-purified, ligated into the pCR4 vector and transformed into TOP10 competent cells (Invitrogen). Inserts were sequenced by the University of Florida, Interdisciplinary Center for Biotechnology Research (Gainesville, FL).

Once oligonucleotide specificity was established, cDNA synthesis and multiplex PCR to detect all three viruses simultaneously were optimized. RNA samples from colonies infected exclusively with each of the three viruses were subjected to quantitative PCR (QPCR) to determine the number of genome equivalents by established methods for SINV-1 (Hashimoto and Valles, 2008a), SINV-2 (Hashimoto and Valles, 2008b), and SINV-3 (Valles and Hashimoto, 2009). RNA from each of these sources was diluted with diethyl pyrocarbonate (DEPC)-treated water to achieve a concentration of 1.5×10^5 viral genome equivalents/μl. Equal volumes of each of the three RNA preparations were mixed together resulting in 50,000 genome equivalents of SINV-1, -2, and -3 in the same tube. RNA purified from worker ants completely free of SINV-1, -2, and -3 was added to this preparation to bring the concentration of RNA to 100 ng/μl. This stock solution was serially diluted with DEPC-treated water to obtain samples with 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, and 5×10^{-1} genome copies of each virus. Each dilution was amended with RNA from non-infected ants (final concentration of 100 ng RNA/μl) to keep the total RNA quantities identical. These RNA preparations were used to optimize cDNA synthesis and evaluate the sensitivity of detection.

cDNA synthesis of the complete mixture of viral RNAs was completed in a 0.5 ml PCR tube containing 1 μl each of oligonucleotide primers (1 μM; p524, p548, p812), 0.5 μl (10 mM) of a dNTP mix, 2.5 μl of H_2O, and 100 ng of total RNA (0.5 μl). This mixture was heated to 65 °C for 5 min in a PTC 100 thermal cycler followed by incubation on ice for 2 min. Then, 2 μl of 5× first-strand buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl, 15 mM $MgCl_2$), 1.375 μl of H_2O, and 0.125 μl of superscript RT (25 units) were added. The mixture was collected by centrifugation and incubated at 55 °C for 30 min, followed by inactivation of the reverse transcriptase by heating to 70 °C for 15 min. cDNA was used as template for multiplex PCR. This reaction was conducted under the following temperature regime, 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s, 68 °C for 30 s and a final 68 °C step for 5 min in a thermal cycler. The reaction was conducted in a 25 μl volume containing 2 mM $MgCl_2$, 200 μM dNTP mix, 0.5 units of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 μM of each primer (p244, p524, p548, p555, p812, and p813), and 1–10 μl of the cDNA preparation (depending on the experiment). PCR products (15 μl unless otherwise noted) were separated on a 1% agarose gel and visualized by ethidium bromide staining. For all experiments, positive and negative controls were run alongside treatments. The positive control was a mixture of SINV-1, -2, and -3 RNAs taken through the cDNA synthesis procedure to multiplex PCR. The negative control was a non-template control also carried through the cDNA synthesis procedure to PCR.

Table 1
Oligonucleotide specificity for the *Solenopsis invicta* viruses.

RNA source	cDNA primer	PCR results with primer set[a]		
		p524/p244	p548/p555	p812/p813
SINV-1	p524	+ (481)	–	–
SINV-1	p548	–	–	–
SINV-1	p812	–	–	–
SINV-2	p524	–	–	–
SINV-2	p548	–	+ (717)	–
SINV-2	p812	–	–	–
SINV-3	p524	–	–	–
SINV-3	p548	–	–	–
SINV-3	p812	–	–	+ (259)
Ant	p524	–	–	–
Ant	p548	–	–	–
Ant	p812	–	–	–

Viral RNA from infected fire ant workers was used as template for the cDNA reaction with each cDNA synthesis oligonucleotide primer. RNA was degraded with RNase and PCR conducted with each oligonucleotide set.

[a] +, successful amplication (amplicon size produced); –, no amplification observed.

The multiplex PCR method was then used to complete a brief survey of SINV-1, -2, and -3 prevalence among randomly collected samples of ant nests from Argentina and the U.S. Samples of *S. invicta* worker ants were collected from field locations in Argentina, the purported source of the *S. invicta* introduction into the U.S. (Caldera et al., 2008), and across the U.S. and evaluated for the presence of SINV-1, -2, and -3. Ant collections were accomplished by plunging a 20 ml scintillation vial into a nest mound for several minutes allowing fire ant workers to fall into the vial. Live ants were returned immediately to the laboratory and total RNA was extracted from 10 worker ants from each nest with Trizol reagent (Invitrogen). A total of 135 and 172 ant colonies were sampled from Argentina and the United States, respectively. RNA from these colonies was used subsequently to conduct multiplex cDNA synthesis and PCR as described above.

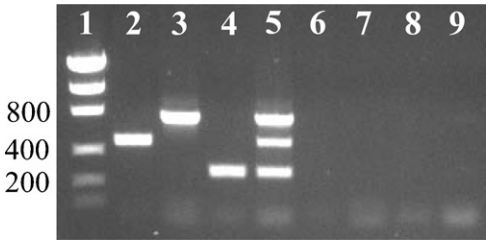

Fig. 1. Specificity of oligonucleotide primers for *Solenopsis invicta* viruses-1, -2, and -3. The cDNA reactions contained an equal number (5×10^4) of genome copies of each of the viruses (as determined by quantitative PCR) and the cDNA oligonucleotide primers p524, p548, and p812. Singleplex PCR was conducted subsequently with oligonucleotide primers p244/p524 (lane 2), p548/p555 (lane 3), and p812/p813 (lane 4). Multiplex PCR was conducted in the reaction represented by lane 5 containing all 3 oligonucleotide primer pairs (p244/p524, p548/p555, and p812/p813). Lane 1 is a molecular weight standard series. Lanes 6 through 9 are negative controls for each corresponding reaction.

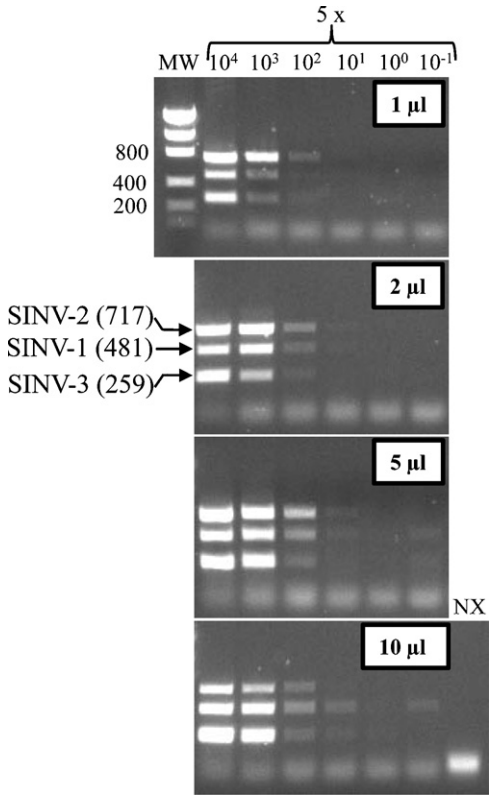

Fig. 2. Detection sensitivity of multiplex PCR to discriminate *Solenopsis invicta* viruses-1, -2 and -3 with varying quantities of cDNA template. MW is the molecular weight standard series. The genome equivalents of the viruses used in the cDNA synthesis are provided at the top. The negative control is designated NX. Increasing volumes of the cDNA reaction were used in multiplex PCR and are indicated by the white boxes.

Table 1 summarizes the specificity of the oligonucleotide primers for each virus genome. cDNA synthesis reactions were completed in an array in which RNA from ants infected individually with each of the three viruses and non-infected ant workers was used as template followed by PCR of each reaction using the virus-specific oligonucleotide primers in separate reactions. SINV-1 yielded an amplicon (481 nucleotides) only when cDNA synthesis was conducted with oligonucleotide primer p524 and PCR with p524 and p244. Similarly, SINV-2 and SINV-3 only yielded amplicons (717 and 259 nucleotides, respectively) when cDNA synthesis was conducted with oligonucleotide primers p548 and p812 and PCR was completed with p548/p555 and p812/p813 oligonucleotide primers, respectively. RNA from SINV-uninfected worker ants did not produce an amplicon with any of these cDNA or PCR oligonucleotide primers. The integrity of the RNA from these ants was verified by successful cDNA synthesis and PCR amplification of a *S. invicta*-specific gene (Valles and Pereira, 2005).

Specificity was confirmed further when multiplexing the cDNA and PCR reactions (Fig. 1). cDNA reactions conducted in the presence of the virus-specific oligonucleotide primers in a single tube with RNA from ants infected with each of the three viruses mixed together resulted in amplification of single amplicons when singleplex PCR was conducted subsequently. Thus, p524 and p244 are specific for SINV-1 (accession number AY634314); p548 and p555 are specific for SINV-2 (accession number EF428566); and p812 and p813 are specific for SINV-3 (accession number FJ528584). Each of these amplicons was excised from the agarose gel, cloned and sequenced. The sequences were identical to the published sequences of SINV-1 (Valles et al., 2004), SINV-2 (Valles et al., 2007a), and SINV-3 (Valles and Hashimoto, 2009), respectively. When the multiplexed cDNA reaction was used as template for the multiplexed PCR reaction with all oligonucleotide primers, three corresponding amplicons were produced (481, 717, and 259 nucleotides for SINV-1, SINV-2, and SINV-3, respectively). No extraneous bands were observed and primer dimerization was minimal (Fig. 1). Negative controls (non-template controls from the cDNA reactions) did not produce amplicons.

Multiple PCR reactions were conducted while varying the oligonucleotide primer concentration. The optimal concentration for the oligonucleotide primers was 0.2 μM. Higher concentrations increased oligonucleotide primer dimerization and decreased sensitivity (data not shown). The sensitivity of the reaction was evaluated by varying the quantity of RNA used in the cDNA synthesis reaction and varying the quantity of cDNA used in the multiplex PCR. Detection of all viruses was consistent down to 5000 genome copies regardless of the cDNA reaction volume used for multiplex PCR (Fig. 2). However, the 5 μl cDNA reaction volume yielded observable PCR amplification of all three virus templates down to 500 copies (Fig. 2).

The multiplex PCR method was employed to conduct a cursory examination of the prevalence of SINV-1, -2, and -3 in *S. invicta* ants in the field in its introduced (U.S.) and native (Argentina) ranges (Table 2). This exercise also provided rigorous field testing and validation of the multiplex PCR method with a large number of samples

Table 2
Prevalence of *Solenopsis invicta* viruses 1–3 in samples collected from across the infected ranges of Argentina and the U.S.

Country	State/province	Collection period	*n*	SINV infection (%)[a]							
				0	1	2	3	1+2	1+3	2+3	1+2+3
United States	Texas	December 2004	63	4.8	4.8	25.4	17.5	17.5	7.9	17.5	4.8
United States	California	April 2005	14	92.9	0	7.1	0	0	0	0	0
United States	Louisiana	April 2005	15	66.7	13.3	0	20	0	0	0	0
United States	South Carolina	June 2005	38	23.7	36.8	18.4	10.5	5.3	2.6	2.6	0
United States	Oklahoma	May/June 2005	25	32	12	0	4	4	4	16	28
United States	Alabama	July 2005	5	0	20	40	20	0	0	20	0
United States	Georgia	June 2005	12	50	50	0	0	0	0	0	0
Argentina	Santa Fe	February 2005	38	10.5	28.9	15.8	7.9	15.8	13.2	5	2.6
Argentina	Santa Fe	February 2009	58	82.8	6.9	3.4	1.7	1.7	1.7	1.7	0
Argentina	Entre Rios	February 2009	15	80	13.3	6.7	0	0	0	0	0
Argentina	Corrientes	February 2009	24	50	25	16.6	0	4.2	4.2	0	0
Summary											
U.S.			172	39 ± 34	20 ± 18	13 ± 16	10 ± 9	4 ± 6	2 ± 3	8 ± 9	5 ± 10
			Probability > *t*	0.6	0.9	0.7	0.1	0.6	0.5	0.1	0.5
Argentina			135	56 ± 34	19 ± 10	11 ± 7	2 ± 4	5 ± 7	5 ± 6	2 ± 2	1 ± 1

[a] 0 = no virus detected; 1 = SINV-1; 2 = SINV-2; 3 = SINV-3; U.S. and Argentinean summary values (±standard deviation) for each virus infection category were not significantly different by Student's *t*-test ($p < 0.05$). The *t*-statistic for unequal variances is provided.

($n = 172$ ant colonies from the U.S., and $n = 135$ ant colonies from Argentina). The U.S. ant samples were collected from December 2004 through June 2005 from across the infested region of the U.S. The prevalence of each virus varied considerably from site to site. For example, some regions exhibited a very low incidence of infection (CA, LA) while in other areas all ant colonies were infected (AL). Argentinean-collected samples exhibited similar incidence patterns, albeit with less variation (based on standard deviation values) compared with U.S. fire ant colonies. However, this could be the result of sampling only during February in Argentina. SINV-1 is reported to exhibit a temperature-correlated incidence in the U.S. (Valles et al., 2007b) and other positive-strand arthropod RNA viruses have shown similar patterns (Plus et al., 1975). Therefore, the Argentina sampling may have been biased by limited sampling. A number of individual colonies exhibited multiple virus infections. However, the multiple SINV infection rate was considerably lower than for single virus infections. It is not known whether individual ants were infected with multiple viruses because 10 ants were pooled and used as the RNA source from each colony. Comparison of viral infections between *S. invicta* colonies in Argentina and the U.S. showed no statistical differences (Student's *t*-test), regardless of category (Table 2).

The relationship between the *S. invicta* viruses and their host, *S. invicta*, is not understood completely. In some cases, SINV-1 infection was associated with colony death, but in others, infection resulted in no apparent symptoms (Valles and Strong, 2005; Valles et al., 2004). SINV-2 was never associated with overt disease symptoms (Valles et al., 2007a) and SINV-3 was nearly always associated with colony death and collapse (Valles and Hashimoto, 2009). These characteristics are similar to the honeybee and the 18 known positive-strand RNA viruses that infect it. Often, viral infections are asymptomatic and persistent and other times, overt symptoms and/or mortality are presented (Chen and Siede, 2007). Characterization of the *S. invicta* viruses is just beginning. The multiplex PCR method described provides the capability to detect the three known *S. invicta* viruses simultaneously in a single reaction. The multiplex PCR method is expected to facilitate epidemiological investigations by identifying when and where SINV infections occur, develop, and spread. Furthermore, virus transmission, development, and spread within the colony structure could be elucidated with the multiplex method. It is important to acknowledge the possibility of false negatives based on a detection limit of 500 genome copies. Thus, incipient viral infections may not be detected.

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